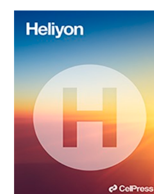
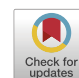
Heliyon 9 (2023) e19710

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon





Selection, alkaline phosphatase fusion, and application of single-chain variable fragment (scFv) specific to NT-proBNP as electrochemical immunosensor for heart failure

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A B S T R A C T

Heart failure has a high global prevalence, with symptoms such as breathlessness, fatigue, and swelling. Early detection is crucial, as the condition worsens over time and can be fatal. This study identified the single-chain variable fragment (scFv) that specifically binds to the heart failure biomarker N-terminal pro B-type natriuretic peptide (NT-proBNP) using biopanning techniques for the development of an alternative diagnostic tool. Ten clones were identified that bound to the target peptide, with two clones (scFv-16 and scFv-36) selected for further analysis. Soluble scFv-16 and scFv-36 were produced and fused with alkaline phosphatase (AP) for potential applications. The binding efficiency and specificity levels of scFv to natriuretic peptides were evaluated using surface plasmon resonance (SPR) analysis. The values of the dissociation constant (K_D) for NT-proBNP of scFv-16, scFv-36, scFv-16-AP, and scFv-36-AP were in the range 3.72×10^{-7}–3.42×10^{-8} M with high specificity. All constructed scFvs had specificity to NT-proBNP, while not binding to A-type (ANP) and C-type (CNP) natriuretic peptides. When AP was combined, the scFv had a slightly higher yield of expression. The enzyme activity of scFv-36-AP was observed first by the absorption at 405 nm at a minimum of 44 nM and then by the naked eye at a minimum of 88 nM. Additionally, the potential application of NT-proBNP binding scFv was preliminarily investigated using an electrochemical technique to directly detect NT-proBNP in phosphate buffer saline. The results revealed the limit of detection at 69.09 pg/mL, which was less than the cutoff value (150 pg/mL) to discharge patients or healthy people. These findings provided promising biomolecules for the development of a reliable and sensitive diagnostic tool for heart failure.

1. Introduction

Heart failure has a global prevalence of 56.19 million cases, as reported by the Global Burden of Disease Study 2019 [1]. It is a condition where the heart is unable to circulate sufficient blood to support the body's needs. This can cause a range of symptoms, including breathlessness, fatigue, and swelling in the legs and ankles [2] that can substantially impact a patient's quality of life. In those with chronic heart failure, symptoms gradually worsen over time, which can ultimately result in acute heart failure [3,4]. Therefore, timely detection of heart failure, before the onset of fatal symptoms is very important [5]. Furthermore, as numbers in the aging population continue to grow, the incidence of heart failure is expected to increase, making early diagnosis even more important

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https://doi.org/10.1016/j.heliyon.2023.e19710
Received 20 May 2023; Received in revised form 22 August 2023; Accepted 30 August 2023
Available online 1 September 2023
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than ever [6,7].

In diagnosing heart failure, echocardiography is commonly used to evaluate heart function and detect irregular heartbeats. However, this requires specialist operators and may be difficult for patients to access. Thus, blood testing methods, such as BNP (brain natriuretic peptide) and NT-proBNP assays, have become popular alternatives [8].

NT-proBNP and BNP are primarily synthesized and secreted from the ventricles of the heart in response to increased myocardial stretching and pressure. Both are derived from proBNP. BNP, a natriuretic peptide, plays a role in regulating blood volume and pressure [9]. On the other hand, NT-proBNP is a non-active fragment of proBNP [10]. ProBNP is a peptide composed of 108 amino acids and contains a region that can be O-glycosylated at Thr36, Ser37, Ser44, Thr48, Ser53, Thr58, and Thr71 [11,12]. This glycosylation allows the peptide to be secreted into the bloodstream. When Thr71 is not glycosylated, proBNP is cleaved into two fragments: the N-terminal fragment (NT-proBNP), which is 76 amino acids long, and the C-terminal fragment (BNP), which is 32 amino acids long [13].

NT-proBNP is a preferred biomarker for diagnosing heart failure because of its longer half-life in the bloodstream, providing greater reliability than BNP [8,9]. The use of NT-proBNP for heart failure diagnosis depends on the clinical setting and patient population. In the acute setting, for patients with acute dyspnea, the recommended NT-proBNP levels indicating heart failure are 450 pg/mL, 900 pg/mL, and 1800 pg/mL for patients under the age of 50, patients between the ages of 50 and 75 and for patients over the age of 75, respectively [14]. Nonetheless, regardless of age, an NT-proBNP level below 300 pg/mL is a very strong indicator that acute HF is unlikely to be present [14]. For patients with NT-proBNP levels below 150 pg/mL, hospitalization or admission to an intermediate care unit or intensive care unit may not be necessary [15].

Several studies have identified NT-proBNP binding molecules using different techniques, with single-chain variable fragments (scFv) being among them. ScFv is a genetically engineered protein that combines the variable regions of both the light and heavy chains of an antibody, linked by a short peptide linker. It retains the binding specificity of an antibody but is smaller and easier to produce [16]. To produce scFv binding NT-proBNP, rabbits are immunized with the recombinant NT-proBNP-Fc fusion protein and total RNA is extracted from the spleen and bone marrow harvested from the immunized rabbits to construct a phage display library of combinatorial scFv, after which the selected scFv is subjected to site-direct mutagenesis to perform affinity maturation [17].

The current research aimed to produce scFv binding NT-proBNP, specifically at the non-glycosylated part, utilizing biopanning techniques to search for bacteriophages in the Yamo I library [18] developed from human serum. The selected scFv was fused with alkaline phosphatase for further applications. Subsequently, bacterial scFv proteins were purified and subjected to SPR analysis to assess the binding efficiency and specificity of scFv to natriuretic peptides (NT-proBNP, ANP, and CNP). The potential application of NT-proBNP binding scFv was preliminarily investigated using an electrochemistry technique to directly detect NT-proBNP in phosphate buffer saline (PBS). The successful selection of NT-proBNP-specific scFv by this research has paved the way for promising alternative applications to quantitate NT-proBNP for the diagnosis of heart failure.

2. Materials and methods

2.1. Selection of C-terminal NT-proBNP-specific scFv using phage library

Scfv selection was performed using a biopanning technique. Biotin-conjugated C-terminal NT-proBNP (51–76 aa) was chemically synthesized to be used as a target. The human scFv Yamo-I phage library was kindly gifted by Professor Montrop Yamabhai, Suranaree University of Technology, Thailand. Briefly, a streptavidin-coated well was prepared in a 96-well plate before setting the experiment up. The biotin-conjugated peptide at 0.5 μg was immobilized onto the streptavidin-coated well. Human serum albumin (HSA) and streptavidin were separately immobilized for subtraction in equal amounts as the peptide target. The immobilization step was carried out at 37 °C for 16–18 h. All wells were thrice washed and blocked with blocking solution (3% skim milk in 0.1% Tween in PBS). After removing the blocking solution, the Yamo-I phage display library ($\sim 10^{11}$ plaque forming units (PFU), 100 μL) was added and incubated at 37 °C for 1 h. Unbound phages were removed based on extensive washing and trypsinization at 37 °C for 1 h. Bound phages were eluted with 100 μL of 0.2 M glycine-HCl pH 2.0 before being neutralized with 15 μL of 1 M Tris-HCL pH 9.2. The eluted phages were incubated with mid-log-phase-cultured *E. coli* TG1. The phage-infected *E. coli* TG1 were grown in Luria broth (LB) containing 50 μg/mL ampicillin. Forty-five colonies of phage-infected *E. coli* TG1 were rescued by adding KM13 helper phage and further incubating at 37 °C for 1 h without agitation. In the presence of 50 μg/mL kanamycin and 20 mM IPTG, phages that displayed NT-proBNP-specific scFv were produced and precipitated using PEG6000/2.5 M NaCl solution. The precipitated phages were reconstituted in PBS and subsequently evaluated for their binding capacity.

2.2. Binding screening of C-terminal NT-proBNP-specific scFv using indirect phage-ELISA

Biotin-conjugated C-terminal NT-proBNP and HSA coated wells were prepared, as described in the previous section. After the blocking step, 10^9 PFU of each of the 45 phages were added into wells containing the peptide target and HSA antigen control. PBS in the absence of phages was used as the control. The reactions were continually incubated at 37 °C for 1 h. Then, the excess phages were removed and washed. Bound phages were probed with anti-g8p M13 monoclonal antibody (1:5000). After 1 h, the wells were washed and incubated with HRP conjugated goat-anti mouse (1:5000). After washing, TMB substrate was added. Then, the product of the HRP reaction was quantified by measuring the absorbance at 450 nm. The binding capacities of each clone and the control were calculated using the fraction of absorption of NT-proBNP over the HSA ratio. Then one-way ANOVA with Dunnett post-hoc analysis was compared with the control.

2.3. C-terminal NT-proBNP-specific scFv gene verification

Promising clones with approximately 2-fold binding capacity were selected for analysis of their nucleotide sequences of the scFv genes. Briefly, *E. coli* TG1 carrying phagemids were cultured in LB broth containing 100 μg/mL ampicillin. The phagemids were extracted using a GF-1 Plasmid DNA Extraction Kit and used as DNA templates. To verify the scFv gene, polymerase chain reactions (PCRs) were performed. LMB3 (5′- CAG GAA ACA GCT ATG AC-3′) and pHEN seq (5′- CTA TGC GGC CCC ATT CA-3′) were used as the forward and reverse primers, respectively. Each PCR reaction was carried out in 10 μL total volume containing: Dream Tap buffer (10×, 1.0 μL), dNTP (2.5 mM, 1.0 μL), forward and reverse primers (5 μM, 1.0 μL each), and Taq DNA polymerase (2.0 U/μL, 0.1 μL). Then, the volume was adjusted to 10 μL with water. For amplification, each PCR sample was pre-denatured at 95 °C for 10 min, followed by 35 cycles of denaturation (95 °C for 30 s), annealing (60 °C for 30 s), and extension (72 °C for 10 s). Final extension was performed at 72 °C for 10 min. The PCR products were analyzed for size using gel electrophoresis (1% agarose gel) and subsequently analyzed for their nucleotide sequences (Macrogen, South Korea). Next, the nucleotide sequences were analyzed for the variable regions using the international ImMunoGeneTics information system® (IMGT®/HighV-QUEST).

2.4. Phagemid transformation

For phagemid extraction, samples of *E. coli* TG1 carrying phagemids of clones 16 and 36 were cultured in LB broth containing 100 μg/mL ampicillin. The phagemids were extracted using a GF-1 Plasmid DNA Extraction Kit and then transformed into *E. coli* HB2151-competent cells using heat shock transformation. Briefly, 1 μL of phagemids was added to 50 μL of *E. coli* HB2151-competent cells and incubated for 30 min on ice. Heat shock was performed at 42.5 °C for 90 s min and then the sample was returned to ice for 5 min. LB broth at 500 μL was added and then shaken at 220 rpm and 37 °C for 90 min. The transformed *E. coli* HB2151 were grown on LB agar containing 100 μg/mL of ampicillin. Then, colony PCR was performed using LMB3 (5′- CAG GAA ACA GCT ATG AC -3′) and pHEN seq (5′- CTA TGC GGC CCC ATT CA -3′) as the forward and reverse primers, respectively. Positive colonies were randomly picked and then mixed with master mix which contained Dream Tap buffer (10×, 1.0 μL), dNTP (2.5 mM, 1.0 μL), forward and reverse primers (5 mM, 1.0 μL each), and Taq DNA polymerase (2.0 U/μL, 0.1 μL). Next, the volume was adjusted to 10 μL with water. All reactions were subjected to PCR that started with pre-denaturation (95 °C/10 min), followed by 35 cycles of denaturation (95 °C/30 s), annealing (60 °C/30 s), and extension (72 °C/10 s). Final extension was performed at 72 °C for 10 min. The size of each PCR product was analyzed based on gel electrophoresis (1% agarose gel).

2.5. Production of soluble scFv

The conditions for scFv expression in *E. coli* HB2151 were optimized by varying the IPTG concentration (0.1 mM, 0.5 mM, 1 mM) and temperature, and time (16 °C for 20 h, 30 °C for 6 h, 37 °C for 4 h). Then, the established conditions were performed on a large scale using 0.1 mM IPTG, 16 °C for 20 h for scFv-16 and 0.5 mM IPTG, 30 °C for 6 h for scFv-36, respectively.

E. coli HB2151 samples were inoculated into 5 mL of LB broth containing 100 μg/mL of ampicillin. The culture was incubated overnight at 180 rpm with shaking at 37 °C. The overnight culture was transferred into 200 mL of LB broth containing 100 μg/mL of ampicillin and 2% (w/v) glucose and incubated at 37 °C and 180 rpm until the OD_{600} reached 0.7. Each culture was centrifuged (7600×*g*, 4 °C, 30 min) to harvest the cell pellet that was then resuspended in 200 mL of fresh LB broth containing 100 μg/mL of ampicillin and optimized for the concentration of IPTG as well as the temperature and time. Then, the culture was harvested using centrifugation at 7600×*g* for 30 min at 4 °C; all the supernatant was discarded. The pellet was resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.1% Triton X-100, 1 mM PMSF). Next, the cells were sonicated on ice for 30 min at an amplitude of 30, followed by centrifugation at 10,000×*g* for 30 min at 4 °C. The supernatant was kept for purification.

ScFv containing a hexahistidine tag was purified using an HisPur™ Ni-NTA Superflow Agarose column (Thermo Fisher Scientific). The resin was equilibrated using buffer A (20 mM Tris-HCl pH8, 300 mM NaCl, 10 mM imidazole). Soluble scFv in the supernatant was added into the column and washed with 10 mL of buffer A. ScFv was collected using gradient elution with buffer B (20 mM Tris-HCl pH8, 300 mM NaCl, 500 mM imidazole) that was mixed with buffer A in a gradient percentage. SDS-PAGE stained with Coomassie blue and Western blot, using HisProbe™-HRP (Thermo Scientific) as the recognition unit, were used to investigate the results of purification. Purified scFv in elution buffer was concentrated and the buffer was changed to PBS (pH 7.4) using a 3 kDa cut off column (Pall Corporation).

2.6. Production of scFv-alkaline phosphatase fusion molecules

The scFv-alkaline phosphatase fusion molecules were produced by cloning scFv-16 and scFv-36 into the pSANG14-3F vector (Addgene, plasmid number 39264) as described by Martin et al. 2006 [19], resulting in scFv-16-AP and scFv-36-AP, respectively, which were transformed into *E. coli* BL21. Selection of the clones was performed as previously described using the universal primers T7 (5′- TAATACGACTCACTATAGGG -3′) and T7 Terminal (5′-GCTAGTTATTGCTCAGCGG -3′), which amplified a product size of 2500 base pairs.

Expression optimization was performed in a similar manner. After testing several conditions, the final conditions for expression were determined as 0.2 mM IPTG at 30 °C for 18 h for scFv-16-AP and 0.2 mM IPTG at 16 °C for 18 h for scFv-36-AP. Following this, large-scale expression and purification of the fusion molecules was carried out. The purification was analyzed using Coomassie blue-stained SDS-PAGE and Western blot against HisProbe™-HRP (Thermo Scientific).

The activity of alkaline phosphatase at the C-terminal of the scFv fusion was evaluated based on the detection of increased product of the pNPP substrate (Thermo Scientific). Samples of scFv-16-AP and scFv-36-AP in the concentration range 1.76 pM–1.76 μM were mixed with pNPP at a 1:1 ratio and incubated at room temperature for 0, 30, and 60 min, followed by detection at an absorption of 405 nm using Tecan Infinite® 200 PRO which has measurement range 0–4 OD. PBS without any scFv was used as the blank control.

2.7. Surface plasmon resonance

The binding kinetics and affinity between the different natriuretic peptides and scFv were measured using an Open SPR 2-Channel Starter Pack R4.2 (Nicoya). The ANP and CNP were purchased from Sigma-Aldrich. NT-proBNP was expressed and purified in the laboratory. Briefly, NT-proBNP vector (Genorise) was trasformed into *E. coli* BL21 and expressed with 1 mM IPTG at 30 °C overnight. The product was purified using an Ni column and digested using enterokinase (Genscript) to release NT-proBNP. Different concentrations of the analytes (ANP, CNP, and NT-proBNP) were injected into the flow-channel and then passed over the scFv-immobilized amine sensor chip (NECTEC, NSTDA). Briefly, the carboxyl groups on the scFv-16 and scFv-36 (at 30 μg/mL) and on the scFv-16-AP and scFv-36-AP (at 60 μg/mL) were activated on ice with a mixture of EDC/NHS (1:1) for 1 h to chemically couple the ligands to an individual sensor chip. Then, activated scFv was immobilized on a sensor chip at 1200–1500 response units at a flow rate of 20 μL/min in PBS buffer (pH 7.4), which led to the formation of a strong covalent amide bond. The excess reactive groups were deactivated by injecting 200 μL of blocking solution (1 M ethanolamine-HCl, pH 8.5). To determine the binding constants, the analytes at various concentrations (0–50 μM) at 30 μL/min and 25 °C were passed over the chip surface in the PBS buffer (pH 7.4). Each chip was washed with PBS until there was no change in signal and before an increased concentration or different type of analyte was applied. The sensorgrams were analyzed using a 1:1 binding model interaction in the Trace Drawer 1.9.1 software (Ridgeview Instruments).

2.8. Electrochemical measurements

Electrochemical measurement and electrode preparation were carried out using a Potentiostat PalmSens4 unit. Graphene oxide (GO)-modified electrodes were prepared on a commercial screen-printed carbon electrode (SPCE; Quasense) with a carbon working electrode (3 mm diameter), a carbon counter electrode, and a silver/silver chloride reference electrode.

GO in PBS at 0.5 mg/mL was sonicated for 1 h. Then, the homogenous solution was dropped on the SPCE electrode and dried at 50 °C for 30 min. Cyclic voltammetry was applied to reduce the GO layer for 5 cycles with a scan rate of 100 mV/s and a potential from −1.5 to 0 V. Then, the SPCE/GO was rinsed with ultrapure water before the working electrode (WE) was incubated in 100 mM NHS in PBS at 37 °C for 30 min and rinsed with PBS. Next, the scFv-36-AP was immobilized on the WE by incubating 22.5 ng/mL of the scFv at room temperature for 30 min. Then, unbound scFv was washed by rising with PBS. The WE surface was blocked with 1 M ethanolamine (ETA) for 10 min at room temperature before rinsing with PBS. Various concentrations of NT-proBNP in the range 0–525 pg/mL were incubated at room temperature for 30 min. The experiments used 4 electrodes for every measurement.

All the measurements were carried out in an electrolyte solution containing 5 mM potassium ferro/ferricyanide solution with 100 mM KCl as the supporting electrolyte. Square wave voltammetry (SWV) was measured from −0.2 to 0.6 V with a step potential of 0.003

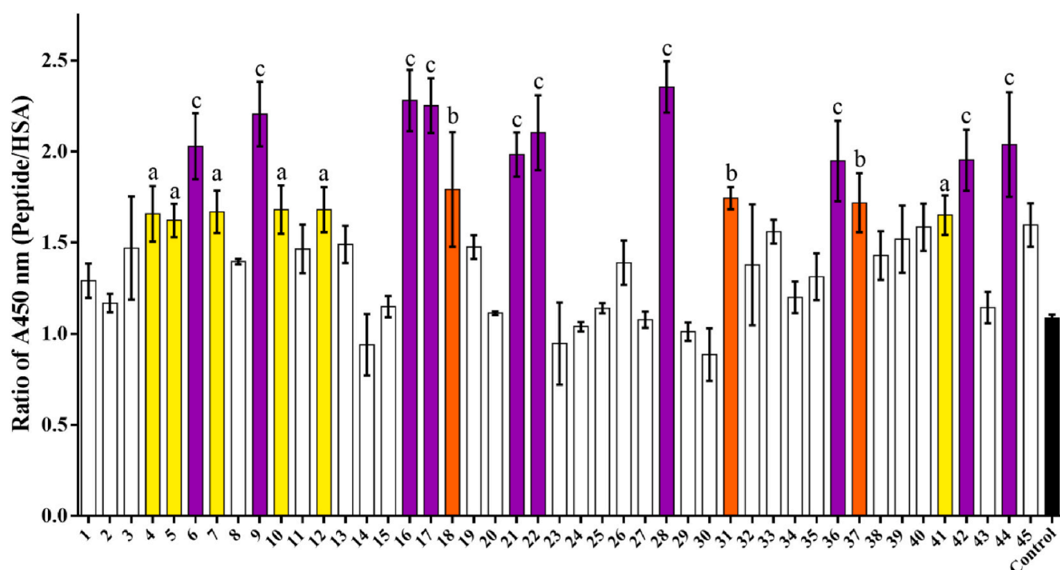

Fig. 1. Binding capacity of phages displaying scFv. Ratio of absorbance at 450 nm of C-terminal NT-pro-BNP to that of HSA for each page is presented on y-axis. Mean ± SD from triplicate experiments is shown by error bars. One-way ANOVA with Dunnett post-hoc analysis was compared with the control. Significant differences are indicated by lowercase letters, where a (yellow), b (orange), and c (purple) represent p values less than 0.05, 0.01, and 0.0001, respectively.

V, an applied amplitude of 0.08 V, and a frequency of 8 Hz. Then, the result was extracted and analyzed using the PSTrace 5.9 software and the peak currents of each step were plotted using the GraphPad Prism software. The limit of detection (LOD) was the concentration of NT-proBNP calculated as the mean of the PBS (blank) plus 3 times the standard deviation (SD) of the blank signal.

3. Results

3.1. Selection and characterization of C-terminal NT-proBNP-specific scFv using phage display and biopanning techniques

The phage display library was used to select C-terminal NT-proBNP-specific scFvs based on a biopanning technique. In total, 45 phages were obtained from the selection of displayed scFvs that were capable of binding the target peptide. To determine the binding capacity of each clone, ELISA was performed and evaluated based on the absorption ratio of C-terminal NT-proBNP-to-HSA. In the absence of phages, PBS had low absorption values for both the peptide target and HSA (A < 0.051). In contrast, scFv-displayed phages had absorption values greater than or equal to 0.520. As shown in Fig. 1, 10 clones (6, 9, 16, 17, 21, 22, 28, 36, 42, and 44) had absorbance values for the peptide approximately twice as high as those for HSA ($p < 0.0001$). Thus, those 10 phagemids were extracted and submitted for sequencing. The nucleotide sequences revealed that clones 16 and 36 had complete variable regions based on IMGT®/HighV-QUEST without stop codons. Thus, they were suitable for further analysis.

3.2. Production of soluble scFv

Phagemids carrying scFv-16 and scFv-36 were transformed into *E. coli* HB2151. Only clones with the correct size of PCR products (approximately 1000 base pairs) were expressed for various conditions. The conditions that scFv-16 was maximumly expressed in soluble form were 0.1 mM IPTG at 16 °C for 20 h, while those for scFv-36 were 0.5 mM IPTG at 30 °C for 6 h. Western blot analysis of the affinity column purification products showed that both scFv-16 and scFv-36 were eluted at 5% and 10% (v/v) buffer B in buffer A (Fig. 2 a–b). However, SDS-PAGE revealed a mixture of other proteins having different sizes from 30 kDa in the 5% buffer B elution but a pure population of 30 kDa proteins in the 10% buffer B elution (Fig. 2 c–d). Thus, scFv from the 10% buffer B elution was concentrated and buffer-exchanged into PBS (pH 7.4). The yields of expression of scFv-16 and scFv-36 were 0.42 and 0.39 mg/L, respectively.

3.3. Production of alkaline phosphatase-fused scFv

The production of soluble scFv-16 and scFv-36 was followed by the generation of their alkaline phosphatase (AP)-fused counterparts, scFv-16-AP and scFv-36-AP, resulting in an increased size of approximately 75 kDa. The optimal conditions for maximum soluble expression of scFv-16-AP were achieved at 0.2 mM IPTG and 30 °C for 18 h, while those for scFv-36-AP were at 0.2 mM IPTG and 16 °C for 18 h. As previously observed, Western blot analysis revealed that the affinity column purification using 5% (v/v) buffer B in buffer A resulted in the elution of scFv-16-AP (Fig. 3a), and scFv-36-AP (Fig. 3b). However, SDS-PAGE showed that pure populations

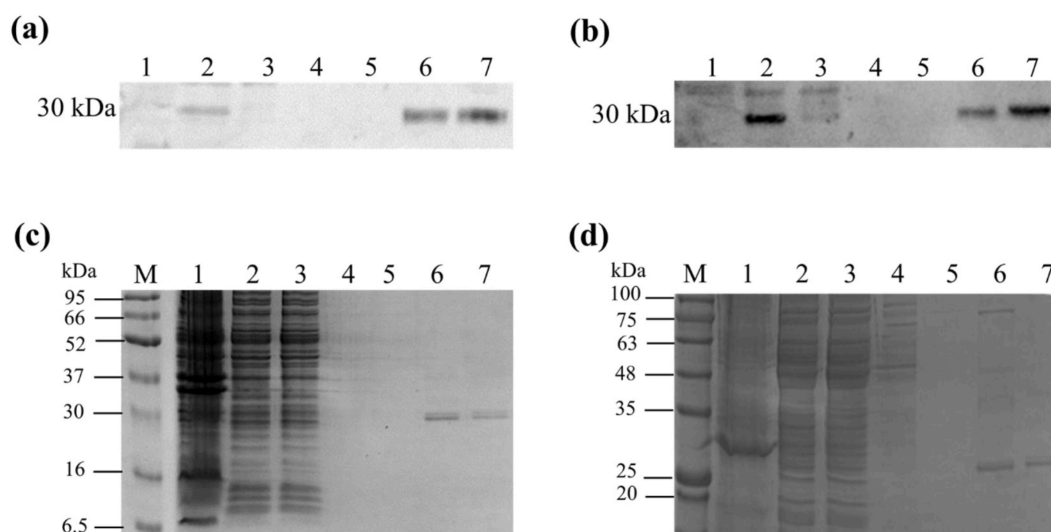

Fig. 2. Western blot and SDS-PAGE analysis of purified scFv. Purification analysis was performed using Western blot against His-tagged scFv-16 (a) and scFv-36 (b), and SDS-PAGE stained with Coomassie blue of scFv-16 (c) and scFv-36 (d). M, markers (kDa); Lane 1, cell pellet; Lane 2, supernatant; Lane 3, flow-through; Lane 4, first washing using buffer A; Lane 5, second washing using buffer A; Lane 6, elution using 5% (v/v) buffer B in buffer A; and Lane 7, elution using 10% (v/v) buffer B in buffer A. Supplementary material 1 a-d have been provided to accommodate non-cropped or unadjusted images of the gels and blots.

of scFv-16-AP (Fig. 3c) and scFv-36-AP (Fig. 3d) was obtained in 20% and 30% (v/v), respectively, of buffer B in buffer A. Then, the scFv-16-AP and scFv-36-AP proteins were concentrated and buffer-exchanged into PBS (pH 7.4), with both achieving a slightly higher yield of expression of 0.5 mg/L.

3.4. Activity of fused alkaline phosphatase

The activity of the AP-fused molecules was evaluated based on the detection of an increase in the yellow product having a maximum absorption at 405 nm. The results (Fig. 4 a) demonstrated that 1.76 μM of scFv-36-AP was substantially more sensitive than scFv-16-AP, with the product immediately observable at 0 min of incubation. When the reaction was incubated for 30 min and longer (Fig. 4 b), activity of scFv-16-AP at 17.6 μM was observed, whereas scFv-36-AP had activity at a lower concentration of 88 nM. Furthermore, at 60 min of incubation, absorption of scFv-36-AP was detectable at 44 nM (A = 0.2999) but not visible to the naked eye (Fig. 4 c). Thus, it could be concluded that the AP activity of scFv-36-AP was more sensitive than that of scFv-16-AP.

3.5. Binding kinetics and specificity of NT-proBNP binding scFv using surface plasmon resonance

The sensorgrams (Fig. 5) showed relatively strong binding of scFv-16, scFv-36, scFv-16-AP, and scFv-36-AP to NT-proBNP at various concentrations, with K_D values in the range 3.72×10^{-7}–3.42×10^{-8} M (Table 1). In principle, K_D is calculated from k_d/k_a, in which k_d and k_a are the dissociation and association rates, respectively. The values of k_a for all scFvs were similar; however, the k_d values of scFv-16 and scFv-36-AP were 10 times lower than the others, implying slower dissociation and thus higher affinity. However, the analysis using a 1:1 binding model interaction could not calculate the affinity of scFvs to ANP and to CNP. This suggested the specificity of scFv to NT-proBNP, with no binding to other natriuretic peptides. The fusion with alkaline phosphatase only slightly increased the binding affinity of scFv-36, shown as a decrease in K_D. Although scFv-16 had the lowest K_D it also had a high SD. Thus, scFv-36-AP was used for further experimentation to preliminarily demonstrate its application for the detection of NT-proBNP using an electrochemistry technique.

3.6. Application of scFv-36-AP as electrochemical immunosensor

A reduction of current was observed due to an increase in the amount of NT-proBNP bound with scFv-36-AP immobilized on the SPCE/GO using SWV (Fig. 6a). The difference between the peak current of the analytes (0–525 pg/mL NT-proBNP) and SPCE/GO after blocking (ETA) is demonstrated in Fig. 6b. The linear range of the change in the peak current was 0–225 pg/mL, based on the formula y = 0.2731x + 33.166, with a coefficient of determination of 0.985 and an LOD of 69.09 pg/mL of NT-proBNP.

4. Discussion

The identification of NT-proBNP binding molecules is crucial for the development of accurate and reliable diagnostic assays for

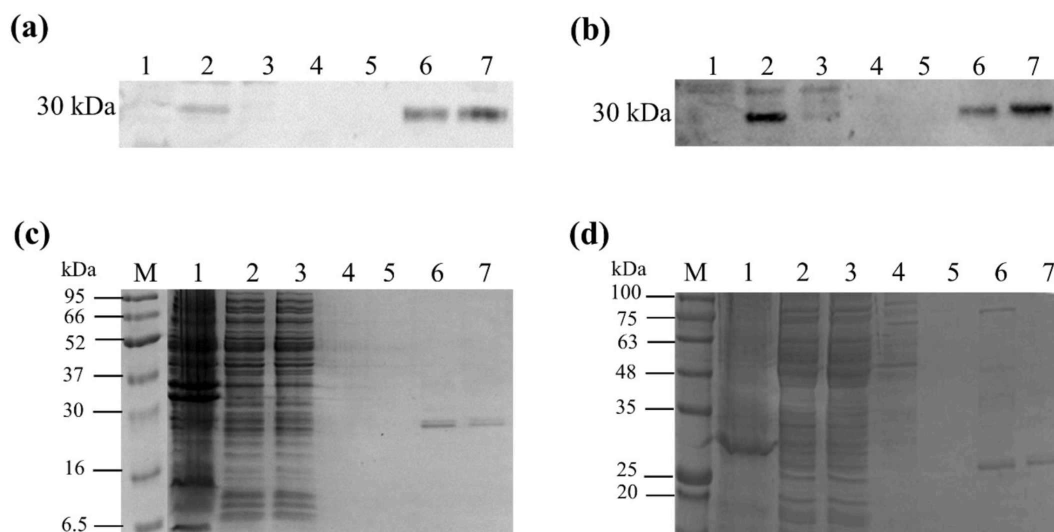

Fig. 3. Western blot and SDS-PAGE analysis of purified scFv-AP. Purification analysis was performed using Western blot against His-tagged scFv-16-AP (a) and scFv-36-AP (b), and SDS-PAGE stained with Coomassie blue of scFv-16-AP (c) and scFv-36-AP (d). M, markers (kDa); Lane 1, cell pellet; Lane 2, supernatant; Lane 3, flow-through; Lane 4, first washing using Buffer A; Lane 5, elution using 5% (v/v) buffer B in buffer A; Lane 6, elution using 10% (v/v) buffer B in buffer A; Lane 7, elution using 20% (v/v) buffer B in buffer A; and Lane 8, elution using 30% (v/v) buffer B in buffer A. Supplementary material 2 a-d have been provided to accommodate non-cropped or unadjusted images of the gels and blots.

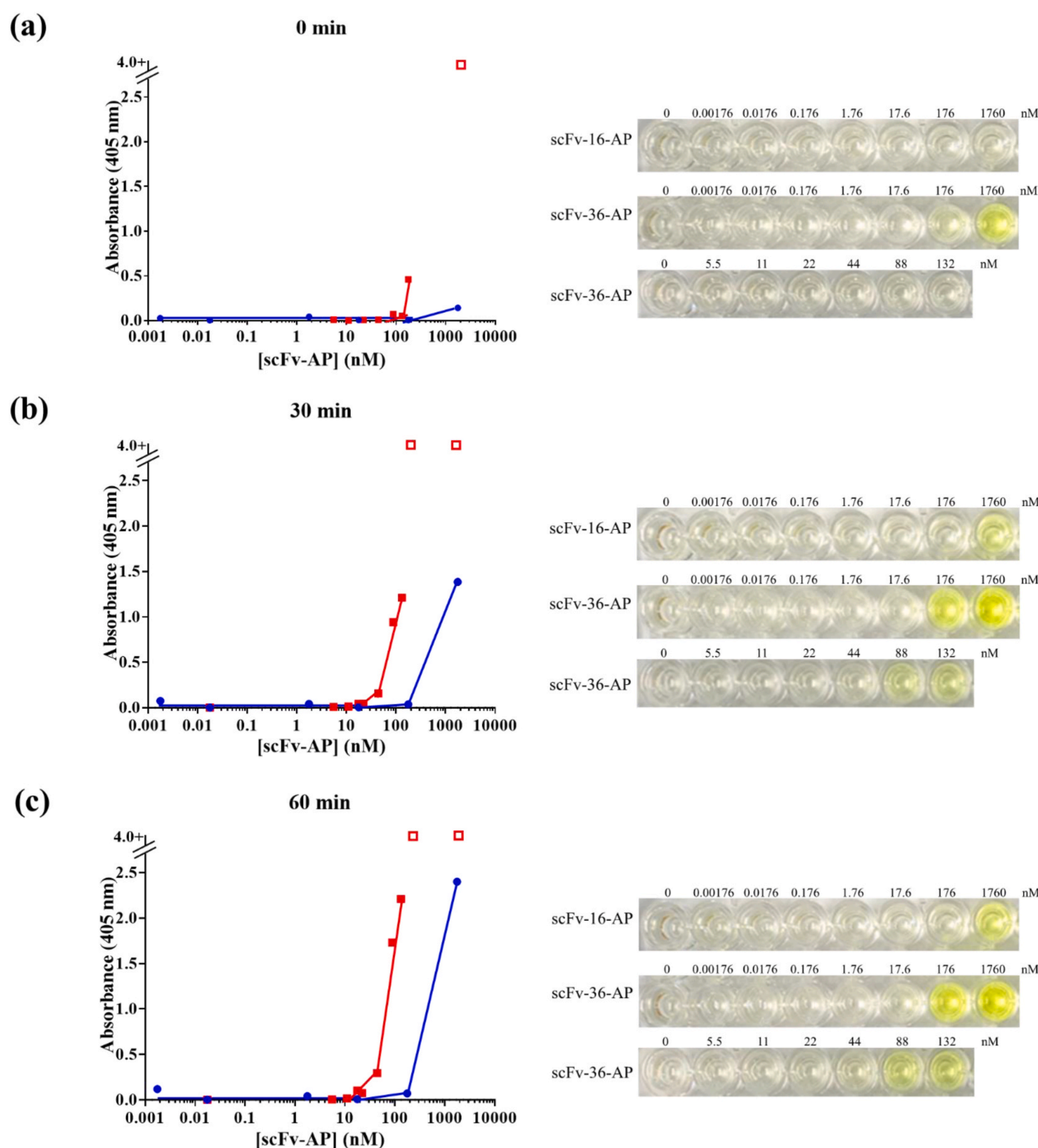

Fig. 4. Alkaline phosphatase activity of scFv-16-AP and scFv-36-AP. Incubation time was 0 min (a), 30 min (b), and 60 min (c). On the left, absorbance at 405 nm was plotted against increasing concentrations of scFv-16-AP (blue circles) and scFv-36-AP (red squares). The open square indicates that the absorption value is too high to measure ($A > 4.0$) based on this technique. On the right, the image of the reactions is shown for observation by the naked eye.

heart failure. Several studies have constructed an NT-proBNP sensor using NT-proBNP binding molecules (which include antibodies [20] and scFv [17]), and using non-natriuretic peptides (somatomedin C) binding aptamers [21]. Both scFv and aptamers have high levels of affinity and specificity for target analytes; however, scFv fragments have an advantage over aptamers in terms of customization. Immobilizing and reactive functional groups can be added to the scFv fragment during the recombinant synthesis process [22], making them highly customizable. In contrast, aptamers require chemical modification to add functional groups, which can be time-consuming and expensive [22].

The current study utilized biopanning techniques and the Yamo I library (a library of non-immunized human scFv [18]) to screen for bacteriophages that could bind specifically to the C-terminal region of NT-proBNP (amino acids 51–76). This region was selected because it is a non-glycosylated part that can distinguish between proBNP and NT-proBNP within the bloodstream. ProBNP is glycosylated at amino acid position 71 [13], which is absent in NT-proBNP, making this region a suitable target for the development of diagnostic assays for heart failure.

Alkaline phosphatase is an enzyme that catalyzes the removal of phosphate groups from molecules, having several applications in biotechnology. For example, it can be fused to scFv to create colorimetric immunotracers for detecting *Loxosceles intermedia* venom

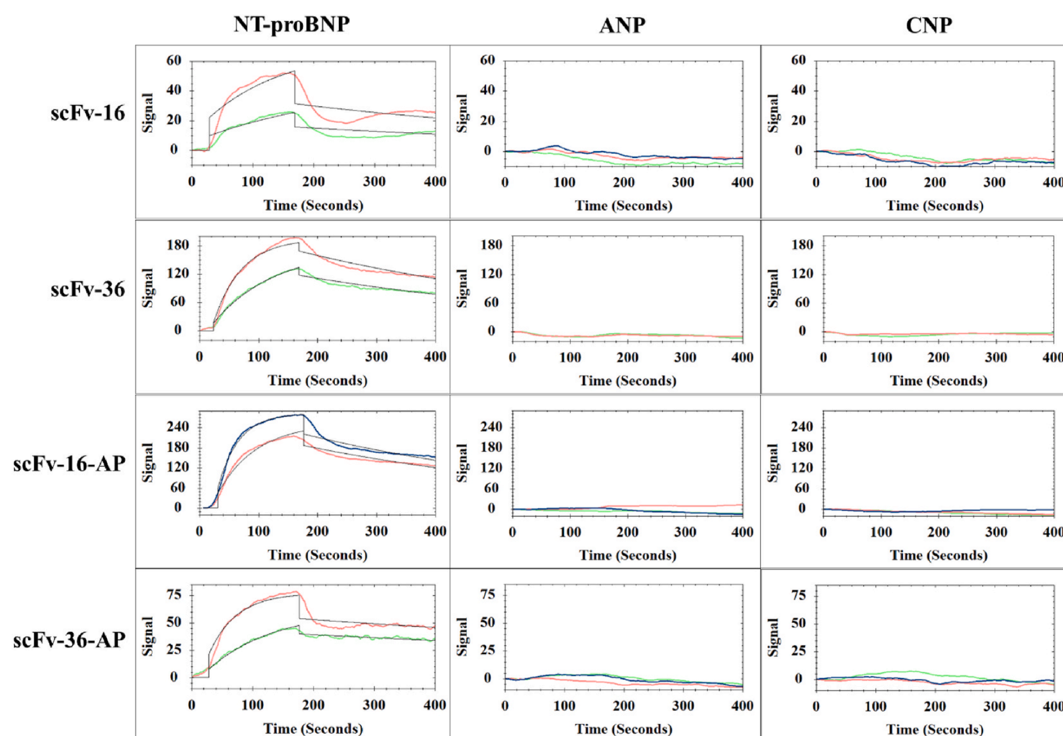

Fig. 5. Binding affinity of scFv and natriuretic peptides using surface plasmon resonance (SPR) analysis. Sensorgrams were plotted using the Trace Drawer 1.9.1 software to show response between scFvs (rows) and NT-proBNP (left-), ANP (middle-) or CNP (right-columns) at concentrations of 1000 nM (green), 2500 nM (red) and 5000 nM (blue).

Table 1
Real-time interaction analysis of scFv-16, scFv-36, scFv-16-AP, and scFv-36-AP.

Ligand	Analyte	k_a (1/(M*s))	k_d (1/s)	K_D (M)
scFv-16	NT-proBNP	$9.6 \times 10^3 \pm 5.0 \times 10^3$	$3.3 \times 10^{-4} \pm 6.3 \times 10^{-5}$	$3.42 \times 10^{-8} \pm 3.35 \times 10^{-8}$
	ANP	–	–	–
	CNP	–	–	–
scFv-36	NT-proBNP	$7.7 \times 10^3 \pm 1.3 \times 10^2$	$1.8 \times 10^{-3} \pm 3.1 \times 10^{-6}$	$2.37 \times 10^{-7} \pm 4.30 \times 10^{-9}$
	ANP	–	–	–
	CNP	–	–	–
scFv-16-AP	NT-proBNP	$5.3 \times 10^3 \pm 5.2 \times 10^2$	$2.0 \times 10^{-3} \pm 4.0 \times 10^{-5}$	$3.72 \times 10^{-7} \pm 4.46 \times 10^{-8}$
	ANP	–	–	–
	CNP	–	–	–
scFv-36-AP	NT-proBNP	$8.6 \times 10^3 \pm 3.03 \times 10^2$	$7.4 \times 10^{-4} \pm 4.32 \times 10^{-5}$	$8.61 \times 10^{-8} \pm 8.10 \times 10^{-9}$
	ANP	–	–	–
	CNP	–	–	–

[23], or used in a sandwich enzyme-linked immunosorbent assay (ELISA) to quantify antigen concentration. In another application, the scFv against AFB1 was genetically fused to alkaline phosphatase, producing a highly sensitive self-indicating reagent [24]. However, the activity of the scFv-AP format can be variable, depending on the clone used. For example, one study reported that the scFv-AP format had superior ELISA activity and higher yields for the collagen VI-specific clone but reduced activity and yield for the anti-desmin clone [19]. The current study yield was 0.5 mg/L of scFv-16-AP and scFv-36-AP, both of which are fusion constructs with alkaline phosphatase, representing a slight increase in yield compared to the non-fused constructs (scFv-16 and scFv-36). Activity sensitivity of AP in scFv-36-AP was to the 44 nM level at 60 min of incubation. This amount was equivalent to 374 ng/mL of NT-proBNP, which is still impractical for the diagnosis of chronic or acute heart failure.

In the current research, other natriuretic peptides—ANP (atrial or A-type natriuretic peptide) and CNP (C-type natriuretic peptide)—were chosen to evaluate the binding specificity using SPR, which has several advantages over other techniques due to SPR being a label-free and real-time technique that enables the quantification of molecular interactions [25]. SPR allows for the measurement of binding affinity and kinetics, which are essential parameters for developing diagnostic assays with high sensitivity and specificity. In the current work, none of the scFvs that were biopanned against the C-terminal of NT-proBNP bound to ANP or CNP. Although the study did not compare the specificity of scFv with BNP, the cross-reactivity between BNP and NT-proBNP is low [26].

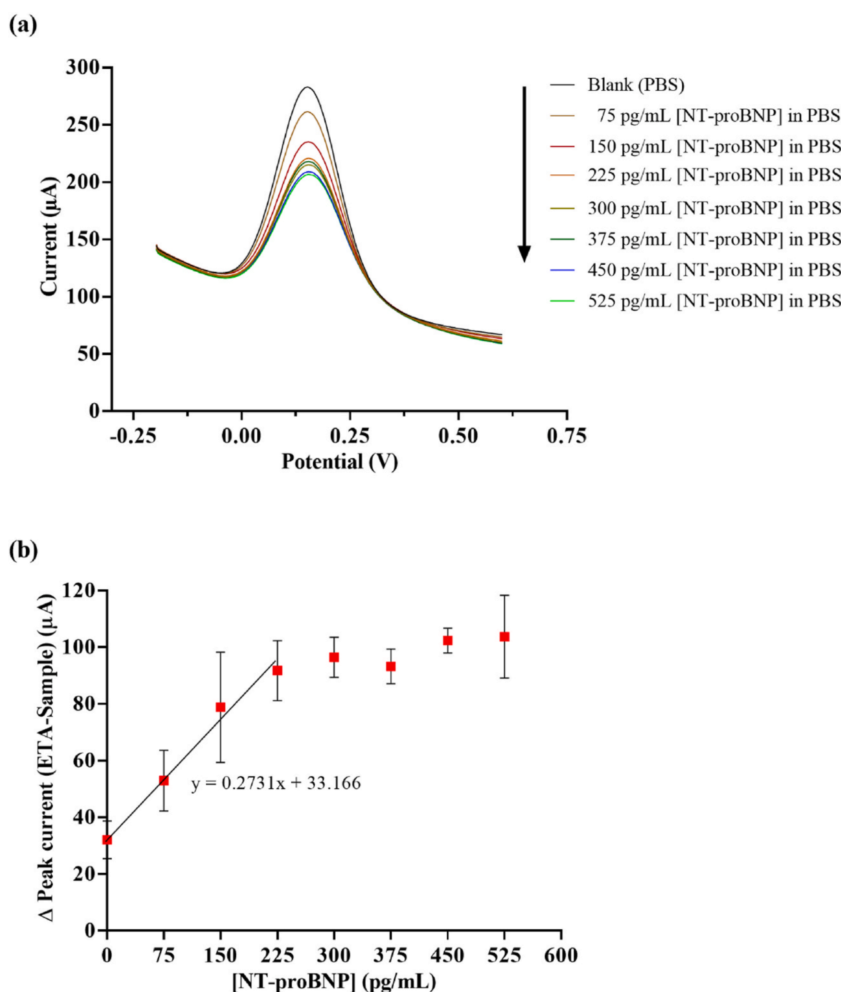

Fig. 6. Application of scFv-36-AP for electrochemical immunosensor. Square wave voltammogram (a) and change of peak current (μA) of 0–525 pg/mL [NT-proBNP] in PBS, compared to ETA (b), where values are means ± SEM of the change of peak current from 4 electrodes.

Therefore, it was considered unlikely that the produced scFv would bind to BNP.

Various techniques have been applied to improve the detection sensitivity of NT-proBNP, including electrochemical methods. An electrochemical method was used in another study, based on a sandwich immunoassay with detection antibodies labeled with AgNP, resulting in an LOD in the range 0.58–2.33 nM of NT-proBNP in serum [20]. Although the linear range (0–225 pg/mL NT-proBNP) reported in the current electrochemical immunosensor was much lower and the LOD of the technique at 69.09 pg/mL was lower than the cut-off value for chronic heart failure [15], detection in various human sera should be investigated as well. Furthermore, the fusion of AP to scFv in the current work (scFv-36-AP) demonstrated the potential to combine the constructed scFv-AP with a competitive electrochemical immunosensor to increase the sensitivity and accuracy when measurement is made in serum samples, similar to the work carried out by Amor-Gutierrez and colleagues [27].

In conclusion, our study identified specific scFvs capable of binding to the C-terminal NT-proBNP using biopanning techniques and used SPR analysis to demonstrate their binding efficiency and specificity to natriuretic peptides. These scFvs have the potential to be used in the development of accurate and reliable diagnostic assays for heart failure, which could facilitate timely detection and treatment, thereby reducing the financial burden on patients and healthcare systems. Further studies are needed to validate the diagnostic potential of these scFv clones and to optimize their performance for clinical use.

Author contribution statement

Sureeporn Wongjard, Pongsakorn Aiemderm: Performed the experiments; Wrote the paper.

Kanchana Monkhang, Kittitat Jaengwang: Performed the experiments.

Lueacha Tabtimmai, Charoenkwan Kraiya, Kiattawee Choowongkomon: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Napachanok Mongkoldhumrongkul Swainson: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was partially funded by the National Research Council of Thailand (NRCT {N34A650492}; The NSRF via the Program Management Unit for Human. Resources & Institutional Development, Research and Innovation {B36G660005}; Graduate Fellowship Program and International SciKU Branding (ISB), Faculty of Science, Kasetsart University, Bangkok, Thailand.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e19710.

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